FONDAZIONE
Monza e Brianza
per il BAMBINO
e la sua MAMMA

UNIVERSITÀ DEGLI STUDI DI MILANO
BICOCCA

Minimal Residal Disease (MRD) as a Surrogate
Enpoint in Acute Lymphoblastic Leukemia (ALL)
Workshop
April 18, 2012

Comparison of flow cytometric and molecular monitoring of MRD in pediatric ALL

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Prerequisites of a reliable technique to detect MRD

a. Sensitivity of at least 10^{-4}, although it depends on the clinical question;

b. *Specificity*, to prevent false-positive results

c. being *quantifiable* within a large dynamic range;

d. *stability* over-time of leukaemia-specific markers, to prevent false-negative results, particularly in long-term studies;

e. *reproducibility* between laboratories (essential for multicenter trials);

f. careful *standardization and quality control* checks;

g. *rapid availability* of results (in time for clinical usefulness)

Planning MRD-based studies

Any MRD technique requires validation according to the clinical setting and specific questions to be addressed: therapeutic scheme, time points, sensitivity requirement, lab expertise, etc...

Guidance for Industry

Bioanalytical Method Validation

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Veterinary Medicine (CVM)
May 2001
BP

Selective and sensitive analytical methods for the **quantitative evaluation of drugs** and their metabolites (analytes) are critical for the successful conduct of preclinical and/or biopharmaceutics and clinical pharmacology studies.

Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use.

The **fundamental parameters** for this validation include (1) accuracy, (2) precision, (3) selectivity, (4) sensitivity, (5) reproducibility, and (6) stability.

Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the method.

FDA: Guidance for Industry
Bioanalytical Method Validation

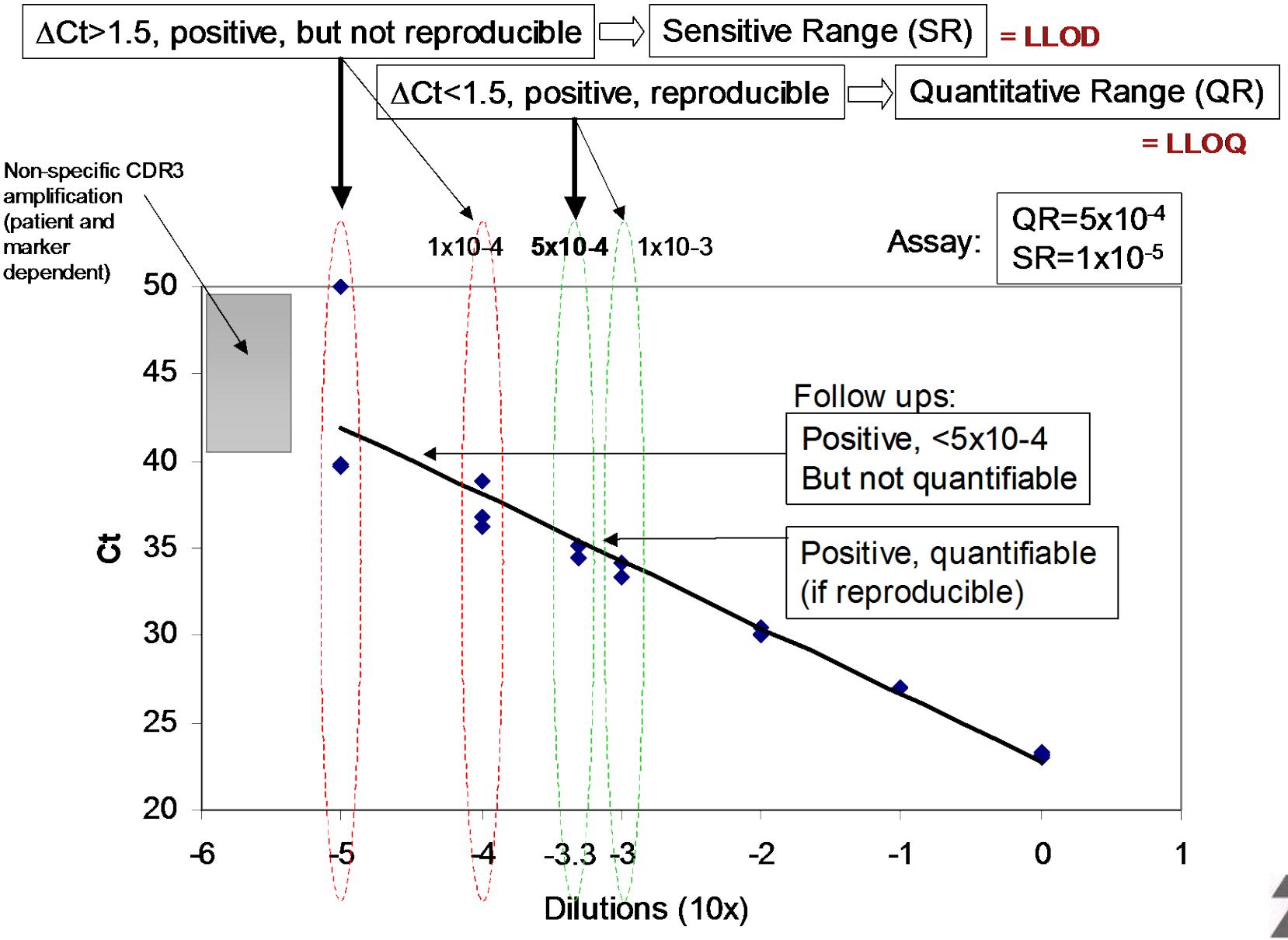
Calibration Curve/Standard Curve/Concentration-Response: A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six to eight non-zero samples covering the expected range, including LLOQ.

Lower Limit of Quantification (LLOQ): The lowest standard on the calibration curve should be accepted as the limit of quantification if the following conditions are met:

- The analyte response at the LLOQ should be at least 5 times the response compared to blank response.
- Analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120%.

Lower Limit of Detection (LLOD): the lowest amount of analyte in a sample that can be detected, but not quantified as an exact value (WHO). Can be expressed by weight, percentage, or calculated copy number, and should be related to independent dilution of specimens with, for example, known microscopic particles in the appropriate specimen matrix, as appropriate.

EuroMRD guidelines



MRD sensitivity/LLOD and quantitative range/LLOQ

Ig TCR DNA strategies usually analyse the equivalent of 2-3.10^E5 cells

Maximum sensitivity = 2-3.10^E-5 = Lower Limit of Detection = **LLOD**

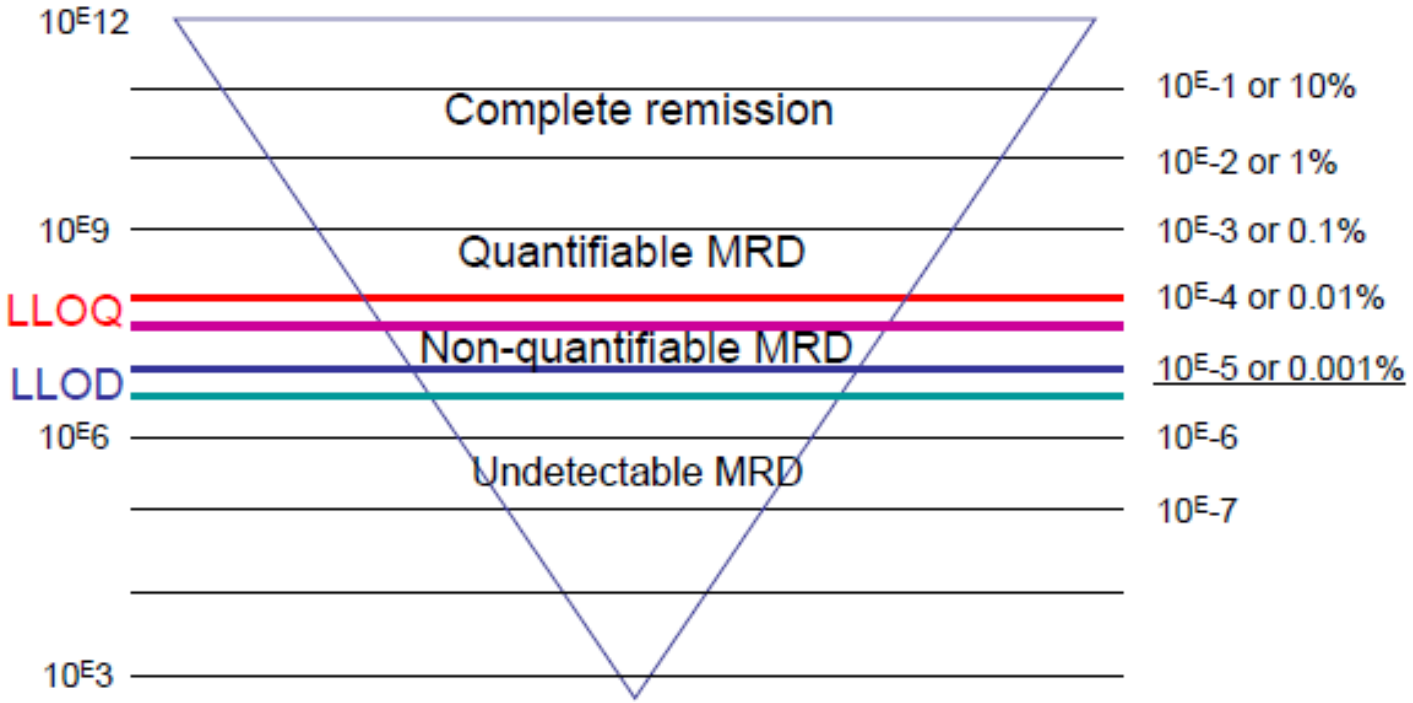
if capable of detecting a single clonal CDR3 for at least 1 target.

Robust sensitivity = Quantitative range (QR) approximately 0.5-1 log higher, depends on ASO = **LLOQ**

MFC strategies usually analyse 2-5.10^E5 cells

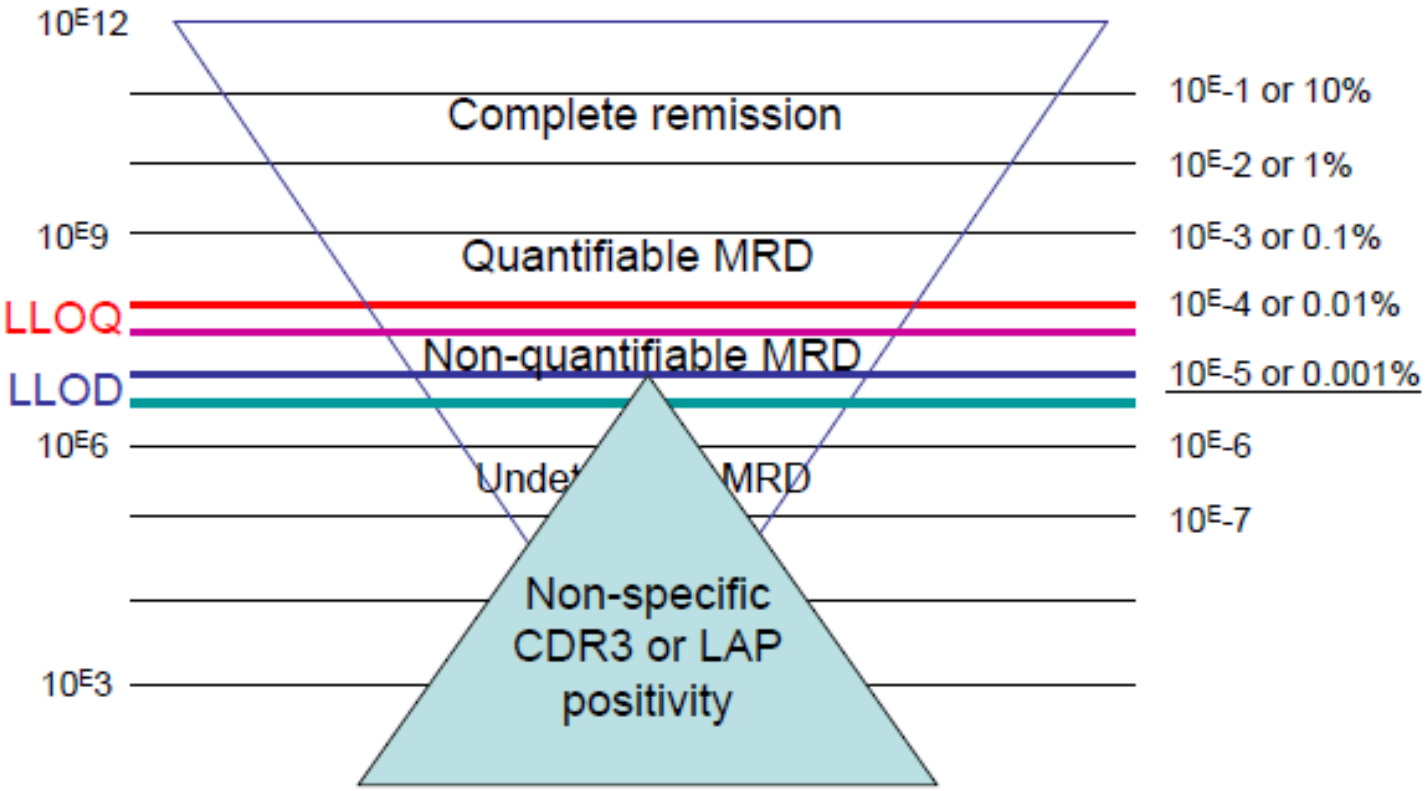
Maximum sensitivity or LLOD = cluster of 10 cells

Robust sensitivity = LLOQ = cluster of 30 cells, ie 10^E-4, but depends on LAP



Courtesy of E.MacIntyre

MRD sensitivity/LLOD and quantitative range/LLOQ
Variable non-specific competitors



Courtesy of E.MacIntyre

RQ-PCR *vs* FCM

	RQ-PCR	***FLOW***
sensitivity	$10^{-4/5}$	10^{-4}
specificity	very high (clone and patient-specific)	high (leukemia-associated)
time of response	slow	very fast
costs *	~ 2500 €	Generally cheaper
standardization	High	Good, but operator dependent
applicability	~ 95 % (of childhood ALL)	>95 % (of childhood ALL)
MRD quantification	DNA (log level)	% or absolute number of cells

* *Complete follow-up per patient*

Studies comparing PCR *vs* FCM

Study	Clinical protocol	n of pts	n of samples	n of TPs	PCR sensitivity	FCM sensitivity	Cut off	Concordance rate
Neale et al Leukemia 199	St.Jude	62	62	1	0.0001%	0.0030%	0.01%	96.7%
Malec M et al , Leukemia 2001	NOPHO 92	23	89	6	0.1% - 0.0001%	0.1% - 0.001%	any level	78.0%
Dworzak and Grumayer, Leukemia and Lymph, 2003	ALL-BFM-95	16	84	> 2	nk	nk	0.01%	83.0%
Veltroni M et al, Haematologica , 2003	AIEOP-BFM 2000	69	69	1	≥0.01%	nk	any levels	95.6%
Neale GAM et al, Leukemia 2004	St.Jude Total XIII; XIV; XV.	227	1.375	3	0.0010%	0.0100%	0.01%	96.7%
Malec M et al, Leukemia 2004	NOPHO 92 + NOPHO 2000	22	71	>2	0.1% - 0.001%	≥0.01%	0.01%	89.0%
Kerst G et al , BJH 2005	nk	30	105	>1	0.1% - 0.001%	≥0.01%	0.01%	97.1%
Ryan J et al , BJH 2008	UK-ALL 97/99	29	151	10	≥0.01%	≥0.01%	0.01%	93.3%

Time Point-Dependent Concordance of Flow Cytometry and RQ-PCR in Minimal Residual Disease Detection for Childhood ALL

Gaipa G, Cazzaniga G, *et al.* (2012)
Haematologica, in press

Characteristics of the study

- Largest number of patients prospectively analyzed according to their routine and independent application
- International Multicenter study (AIEOP-BFM-ALL)
- Centralized national collection of samples
- Comparison between different time points
- Consistent monitoring along time points
- Comparison between Ficoll-based MNC and total NC starting material
- Outcome of MRD discordant patients

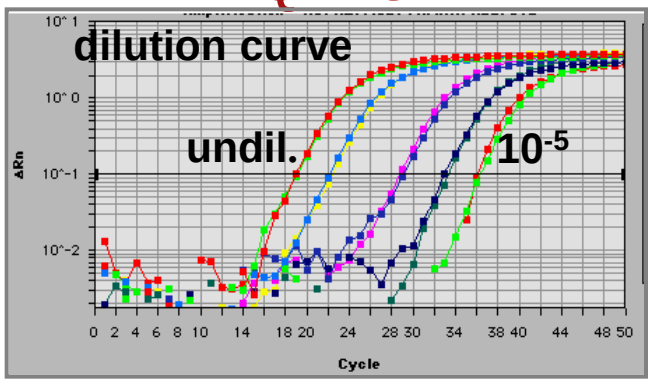
Patients and Samples

- Accrual: from September 2000 to June 2006 in the AIEOP BFM-ALL 2000 trial (Berlin, Monza, Padova, Vienna).
- Eligible and analyzed patients: 4,827
- Analyzed for both PCR and FCM:1,115
- Analyzed samples 2,701 (471 at day 15; 1,115 at day 33; 1,115 at day 78)

Selection was based only on available samples for both PCR and FCM.

Methods

RQ-PCR



Sample processing:

DNA from mononuclear cells (Ficoll).

Analysis

RQ-PCR settings and interpretation were according to the guidelines of EuroMRD-ALL.

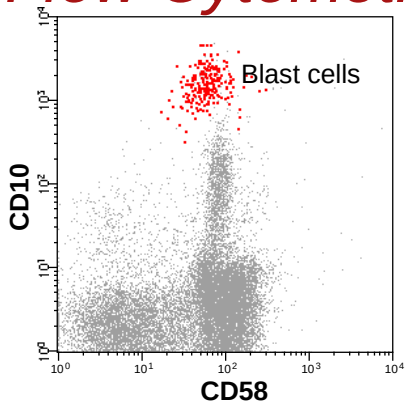
(van der Velden VH et al., Leukemia 2007)

Sensitivity :

At least two Ig/ TcR sensitive markers (≥10^{-4}) per patient were required. *(Flohr T. et al., Leukemia 2008)*

MRD = Log-step reduction relative to the evaluation at diagnosis.

Flow Cytometry



Sample processing:

Whole blood/staining, lyse and wash.

Analysis

FCM settings and interpretation according to standardized methods (4-colors).

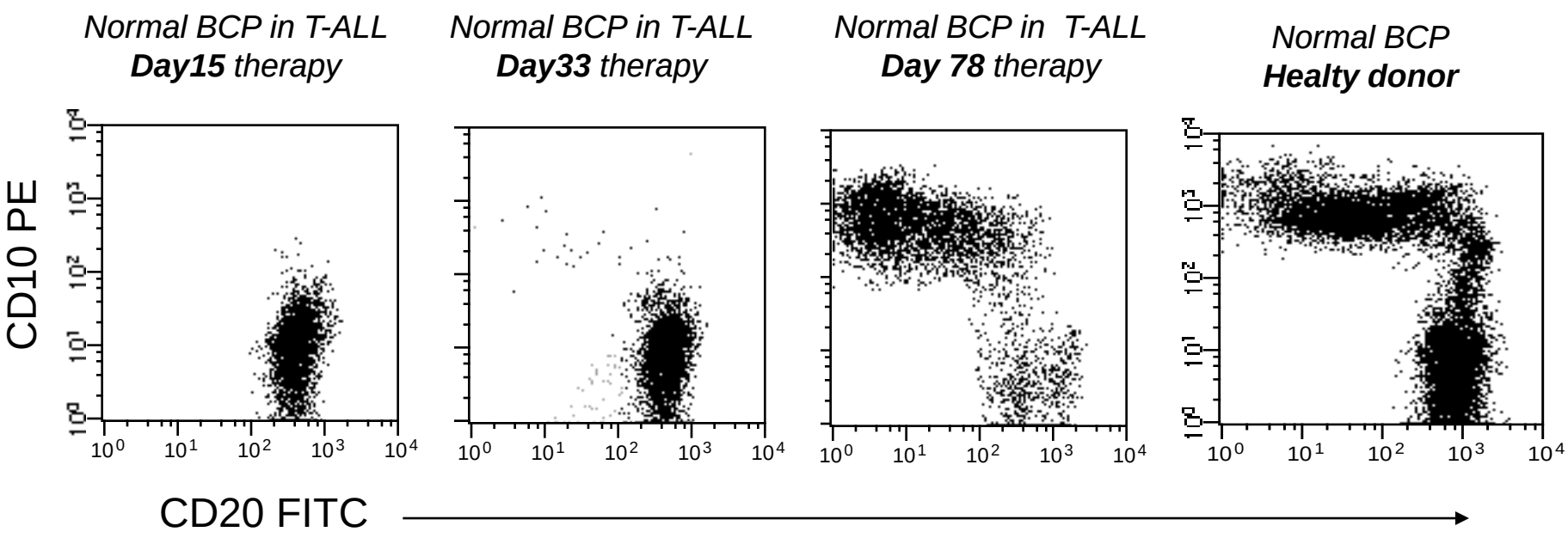
(*Dworzak MN et al., Cytometry B, 2008)*

Sensitivity :

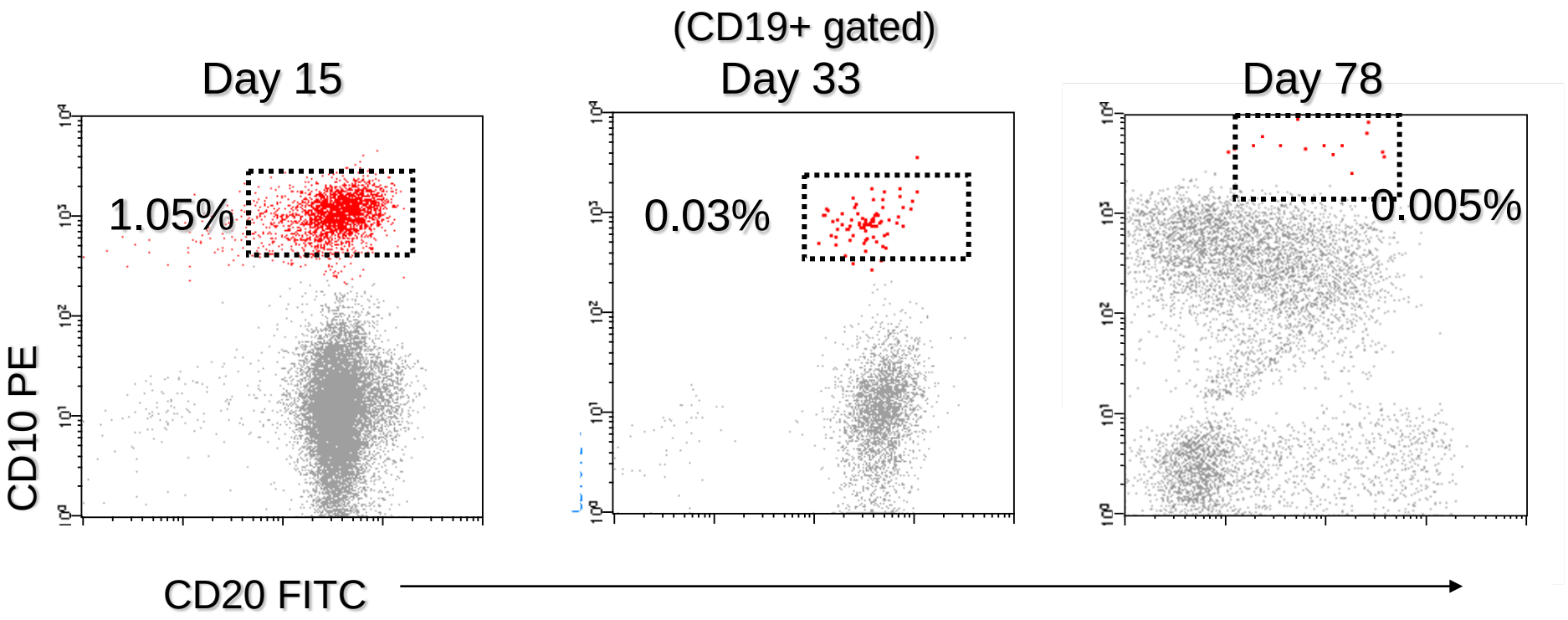
3x10^5 acquired events enabling detection of About 1 leukemic cell among 10,000 normal cells.

MRD = % of blasts on total NC.

Time point-related variables for FCM: cellular background



Time point-related variables for FCM: prevalence of MRD

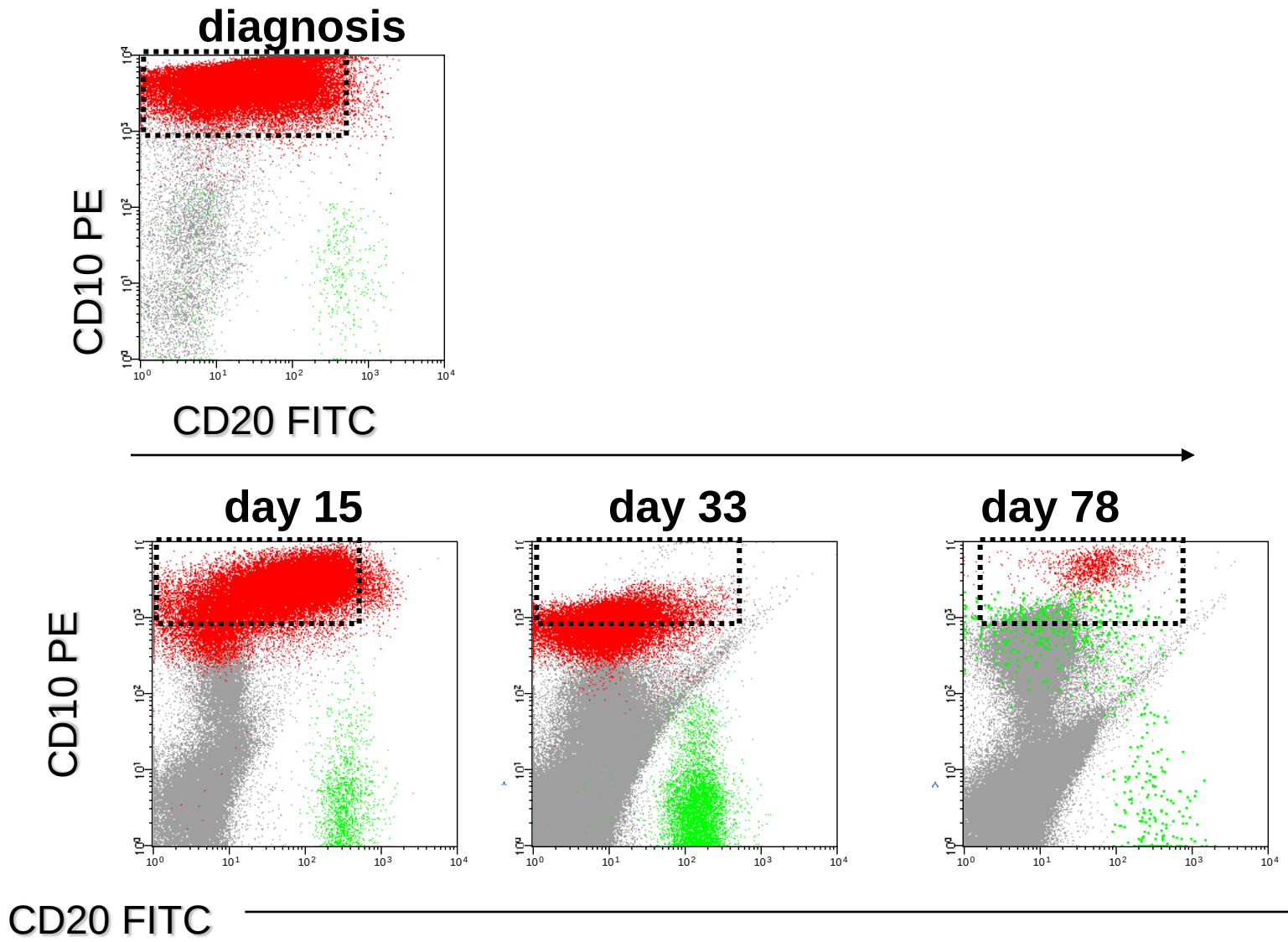


Median **0.58%**
range 0.01%-80.0%
n = 394

Median **0.07%**
range 0.01%-93.0%
n = 303

Median **0.023%**
range 0.01%-53.8%
n = 62

Time point-related variables for FCM:
transient drug-induced immunophenotypic modulation



Dworzak MN *et al.* Cytometry B 2010

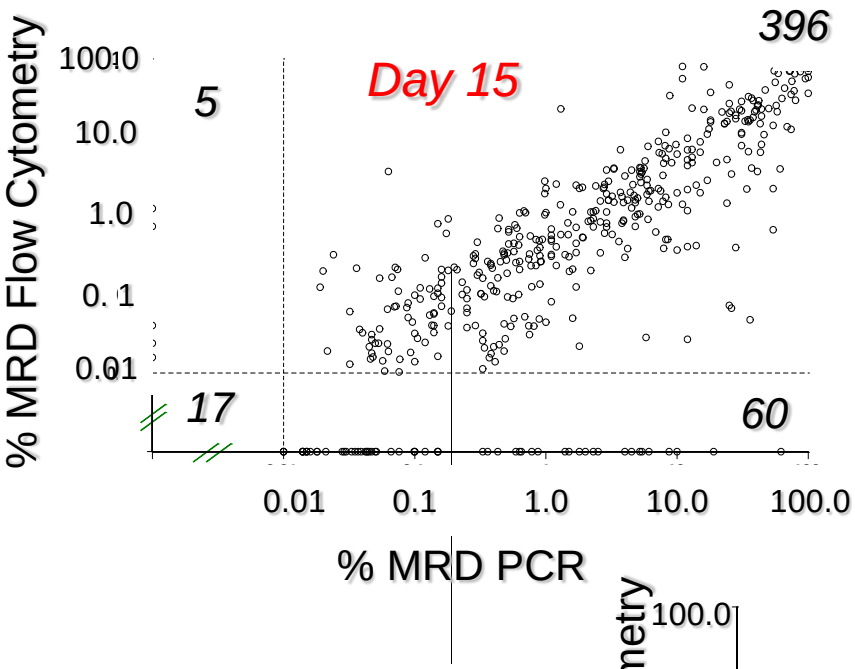
Concordance in MRD detection at each Time Point

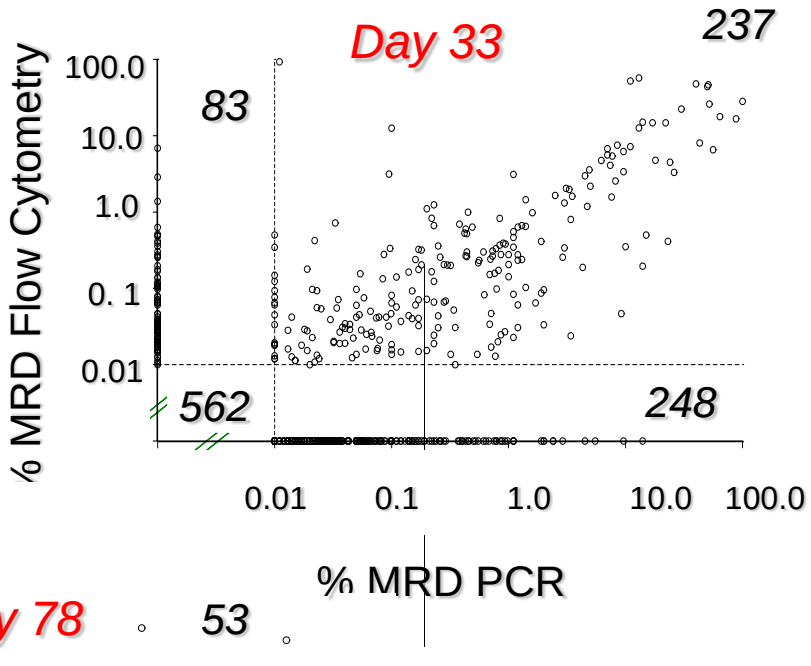
	Day 15 (n of samples)			Day 33 (n of samples)			Day 78 (n of samples)		
	PCR ≥0.01%	PCR <0.01%	Total	PCR ≥0.01%	PCR <0.01%	Total	PCR ≥0.01%	PCR <0.01%	Total
FCM≥0.01%	388	6	394	223	80	303	48	14	62
FCM<0.01%	60	17	77	248	564	812	133	920	1053
Total	448	23	471	471	644	1115	181	934	1115
FCM sensitivity	388/448 = 87%			223/471 = 47%			48/181 = 27%		
FCM specificity	17/23 = 74%			564/644 = 88%			920/934 = 99%		
Concordance	405/471 = **86%**			787/1115 = **70%**			968/1115 = **87%**		

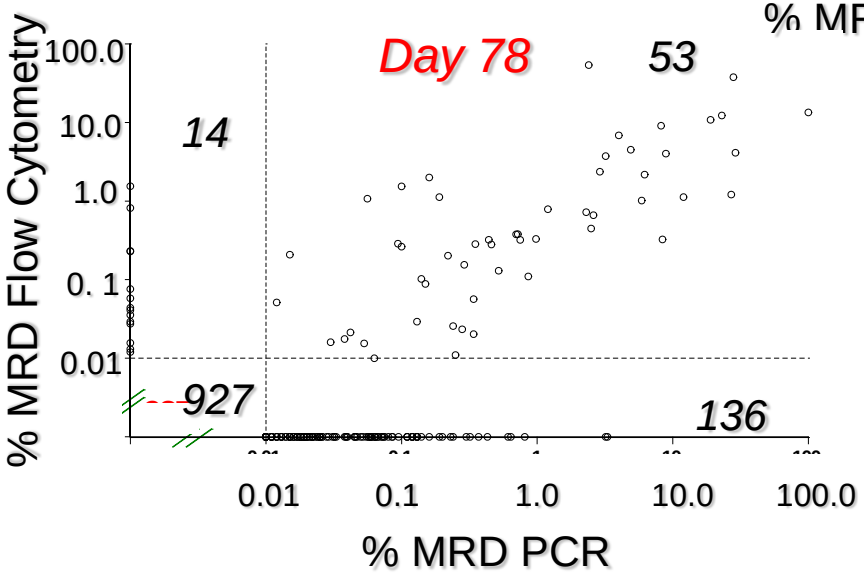
Overalll concordance 80 %

Threshold for positivity ≥ 0.01%

Direct comparison of MRD estimates by PCR and FCM at each time point

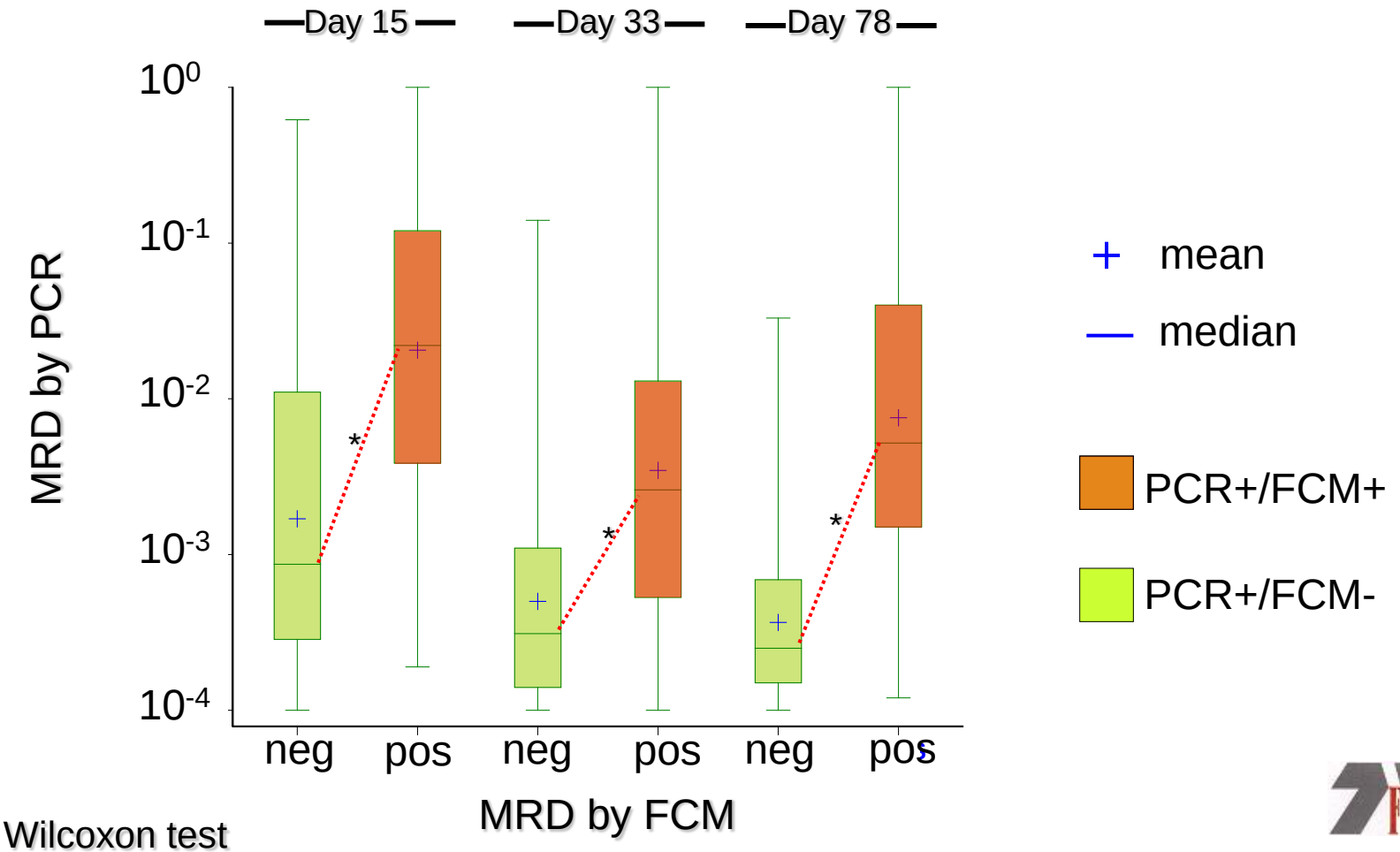






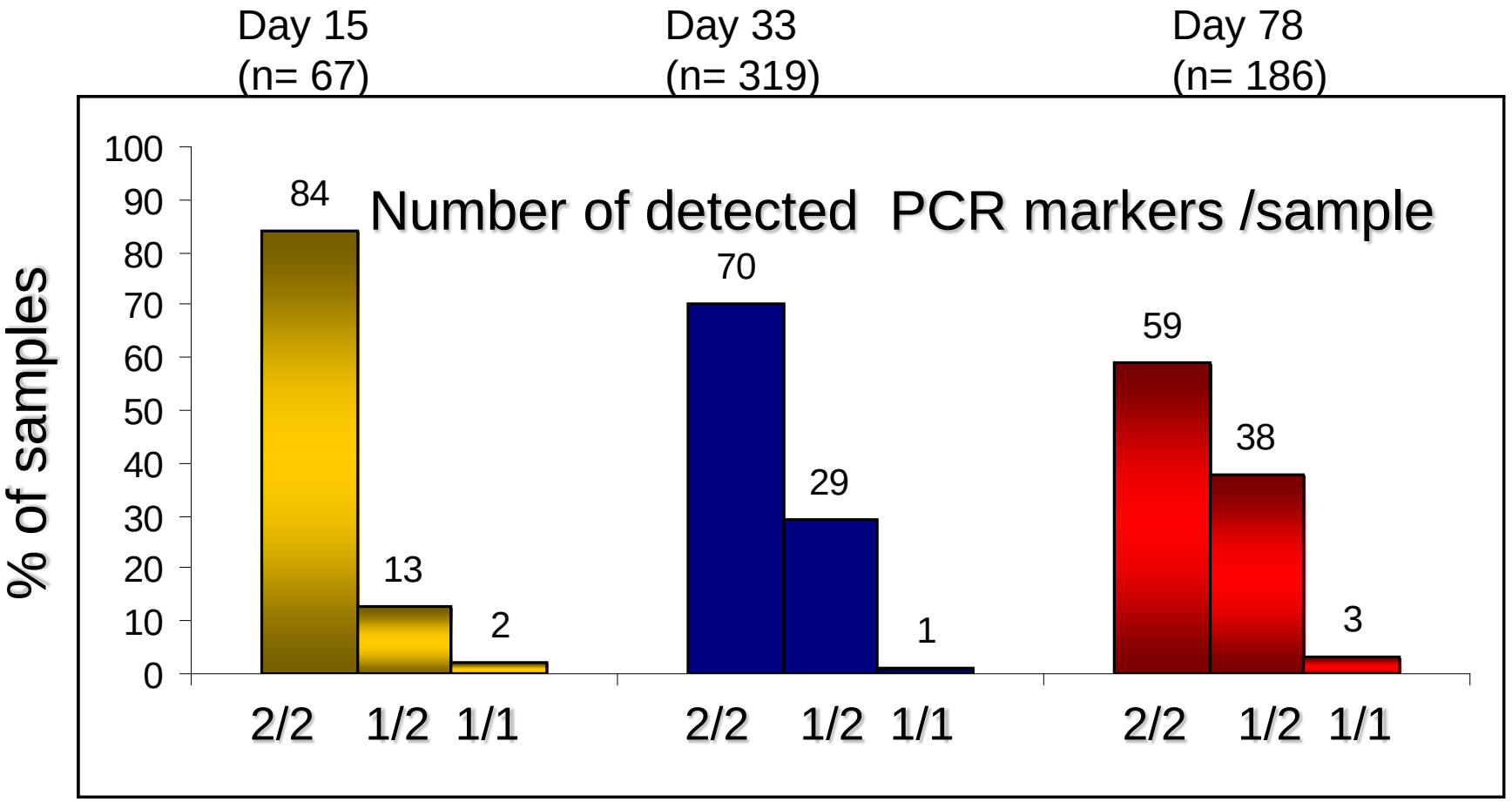
Levels of PCR-MRD in concordant and in discordant samples

At each time point, discordant samples (with FCM <0.01% but PCR ≥0.01%) tended to have a significantly lower positivity level by PCR (10-4 log-range) compared to concordant samples (p<0.001).

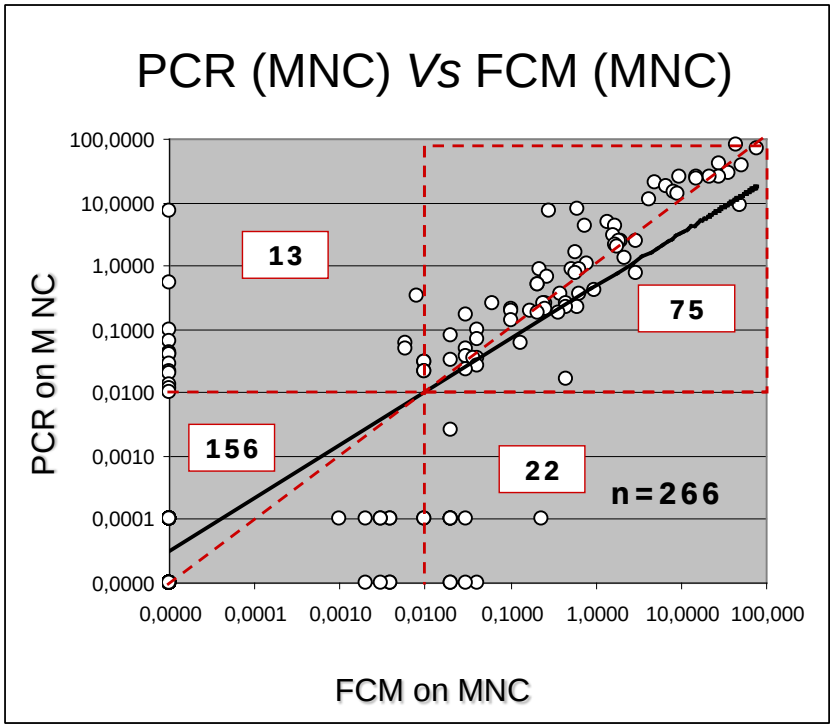


* p<0.001 Wilcoxon test

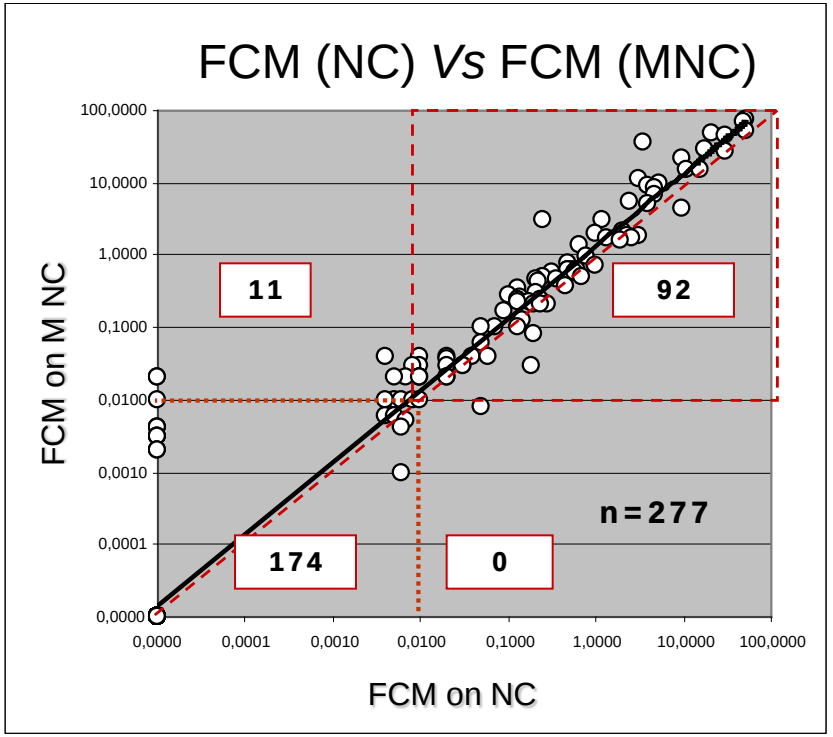
Number of detected PCR markers in PCR+/FLOW- samples



Impact of Starting Material: MNC *vs* total NC



Concordance ***87%***

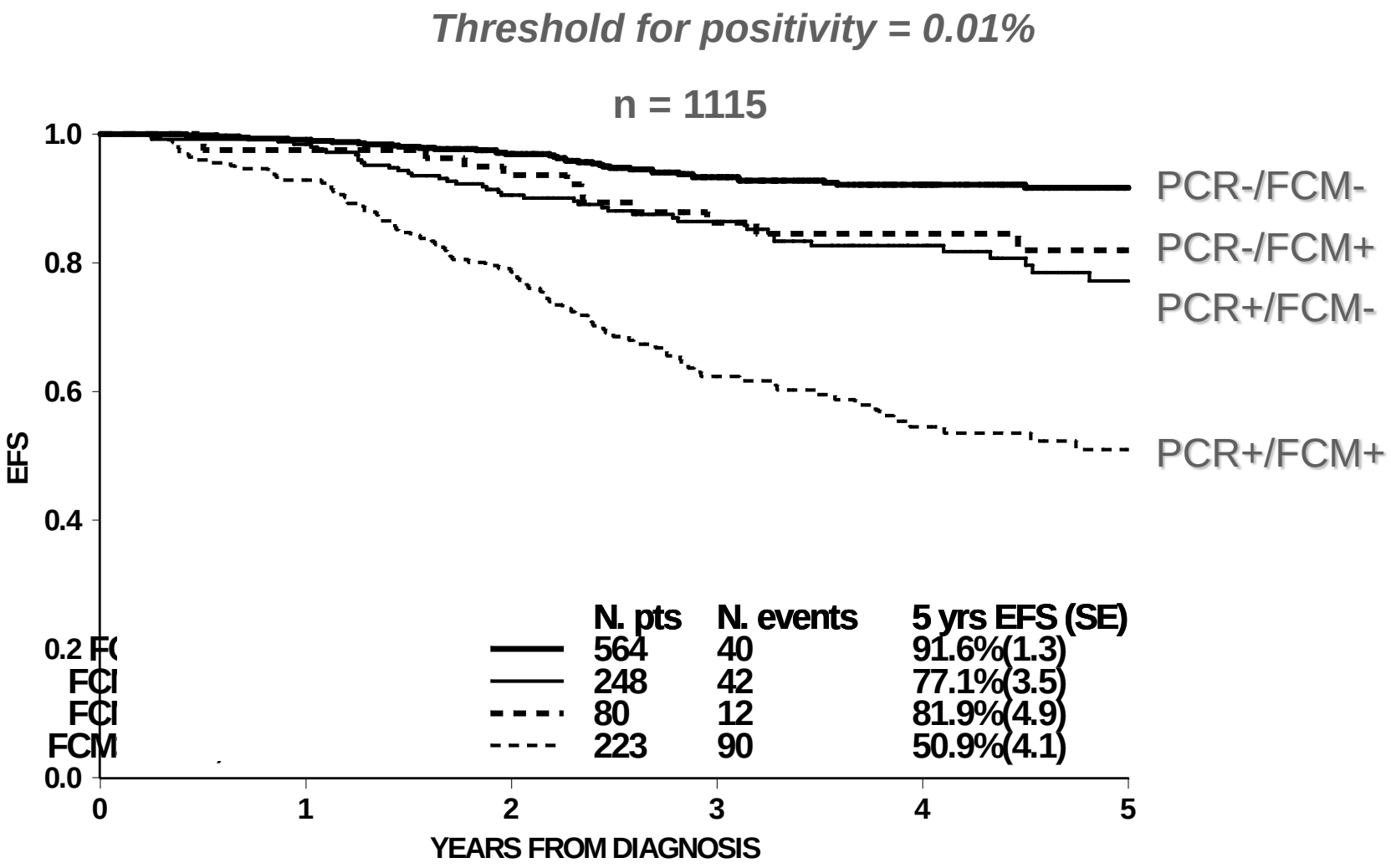


Concordance ***96%***

110 patients (96 with BCP-ALL and 14 with T-ALL)

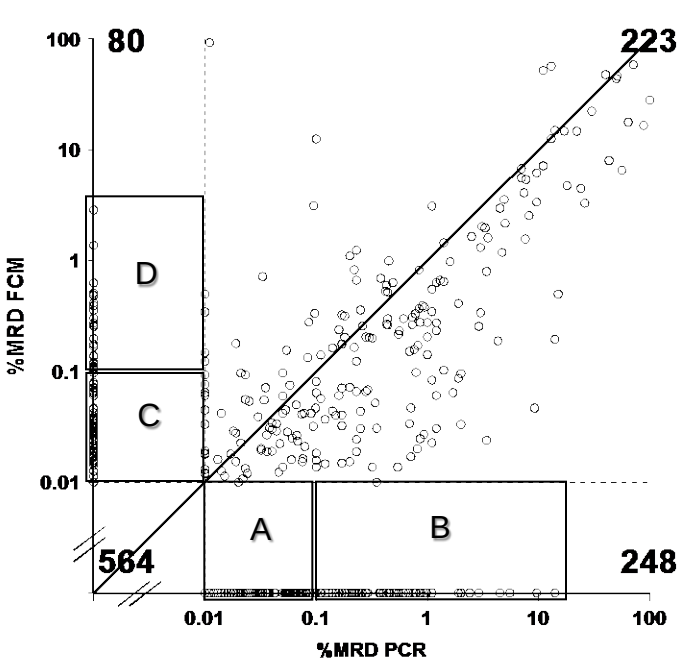
From M.Dworzak laboratory (Vienna)

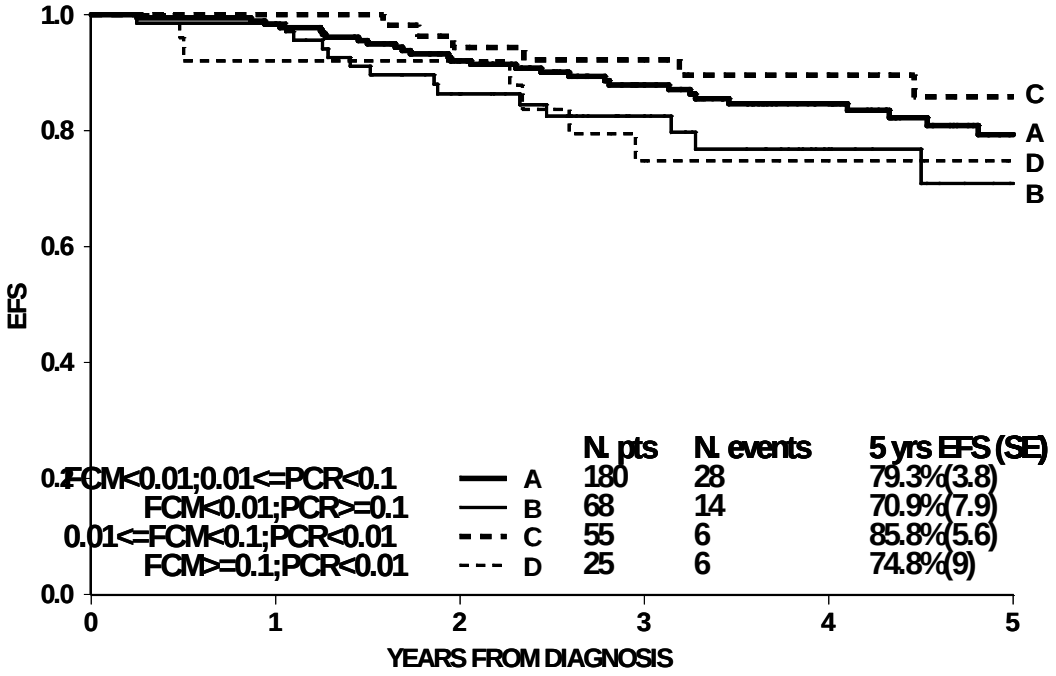
Outcome According to Concordant or Discordant MRD Results at day 33



Outcome for Discordant MRD Results at day 33

Threshold for positivity = 0.01%
N=328





Conclusions (methods)

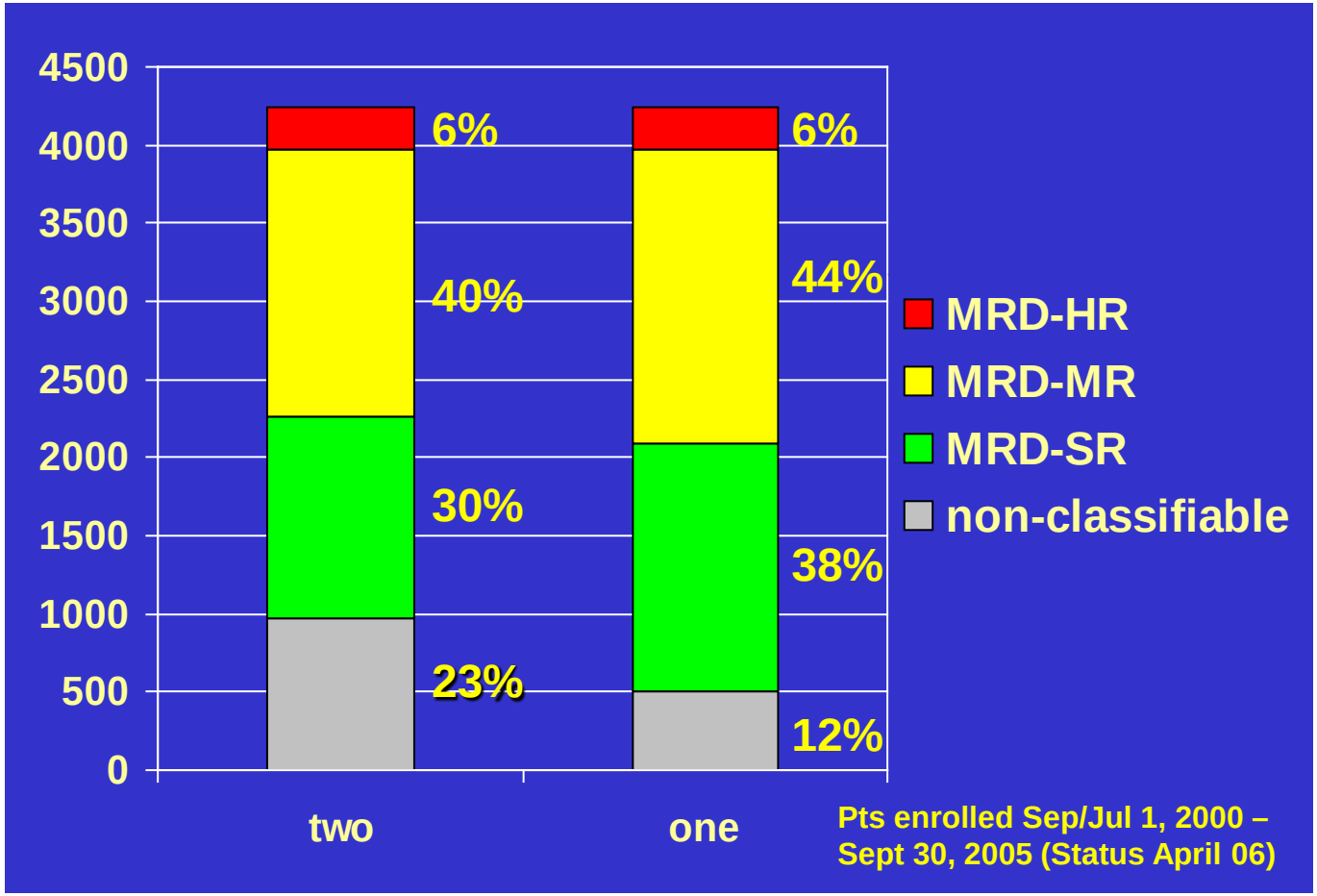
- The overall concordance rate was 80%. Discordances were more frequently due to FCM-negative in samples positive by PCR, occuring at the lowest levels of MRD;
- Minimal impact of using either MNC or NC;
- FCM vs PCR concordance largely depends on the time point, due to: varying regenerating cell backgrounds, phenotypic modulation, diverse tumor burdens
- The two methods measure different cellular targets, then complete quantitative concordance is unlikely.

Conclusions (outcome)

- The intermediate outcome observed in discordant groups may represent the effect of intermediate levels of MRD, at the limit of the sensitivity of both FCM and PCR
- This suggests a potentially complementary role of the two technologies for further improvement of treatment tailoring.
- FCM and PCR retain an independent ability to assess patient risk stratification (Basso G *et al. J Clin Oncol, 2009*; Conter V *et al. Blood,2010)*
- The choice of the methods to be used depends on the expertise, resources and the established clinical trial's design (reviewed in Cazzaniga G *et al. BJH, 2011)*

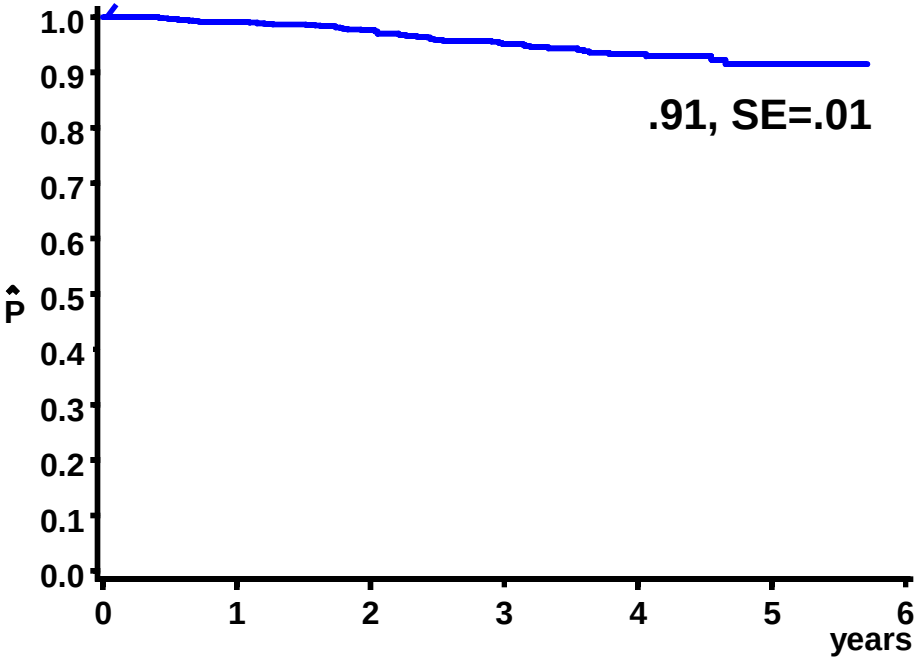
AIEOP-BFM ALL 2000: Comparison of MRD stratification by two or one sensitive marker

n=4239



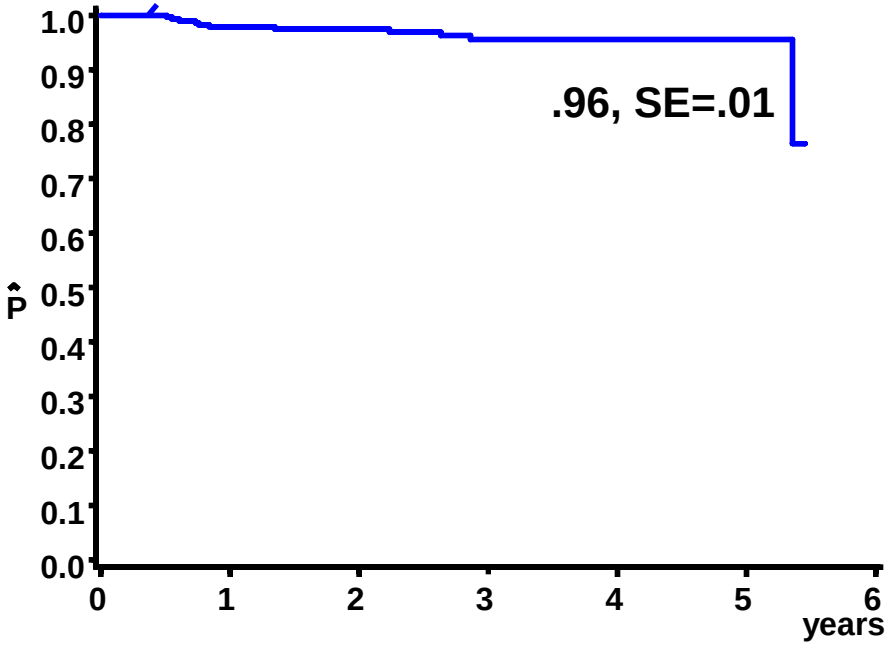
AIEOP + ALL-BFM 2000, EFS (5 years)

MRD-SR 2000
(at least 2 sensitive markers, TP1 + TP2 negative)



N=1290, 52 events

MRD-MR 2000
(only 1 sensitive marker, TP1 + TP2 negative)

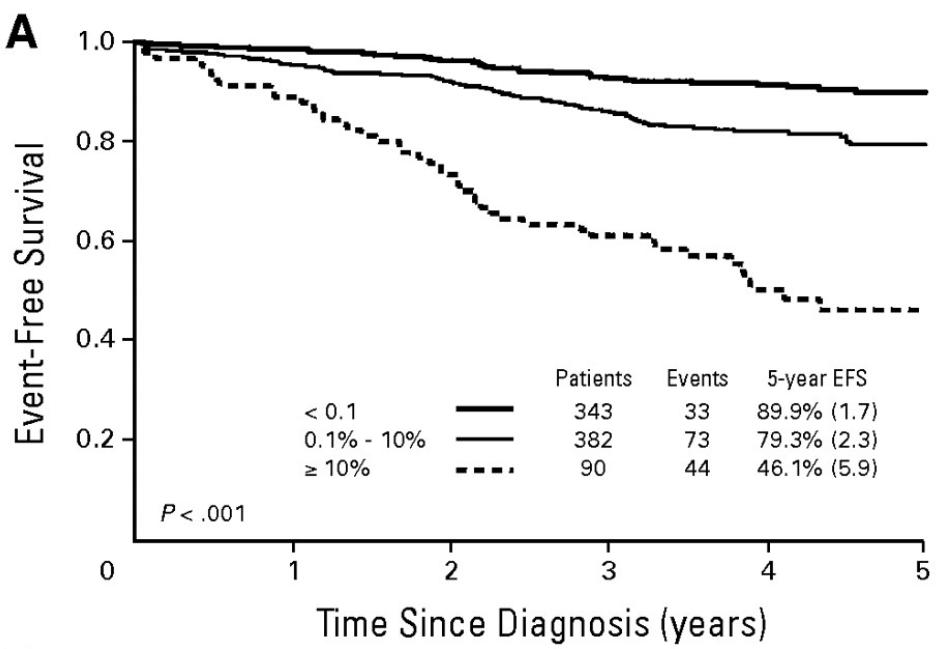


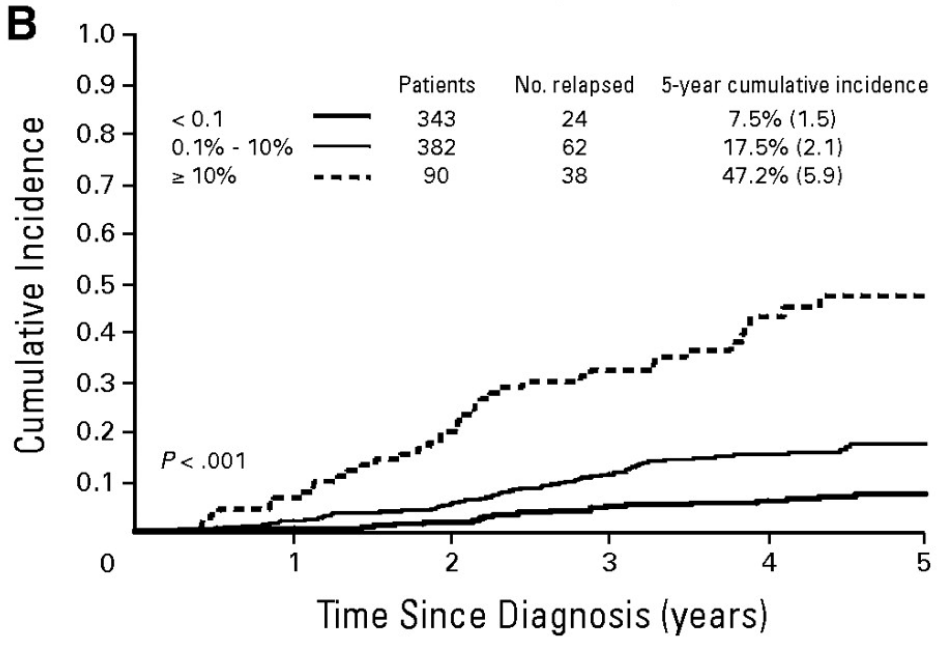
N=300, 11 events

Pts enrolled Sep/Jul 00–Sept 05 (Status April 06)

AIEOP ALL 2000, no clin. HR by FCM at day +15

815 patients





Basso G *et al. J Clin Oncol, 2009*

Stratification by FCM-MRD d15 in AIEOP-BFM 2009

Stratification of the 12% patients who are not stratifiable by PCR-MRD (and without classical HR criteria) according to FCM on day 15:

FCM-MRD d15	
<0,1%	→ SR
≥0,1% and <10%	→ MR
≥10%	→ HR

Aknowledgments

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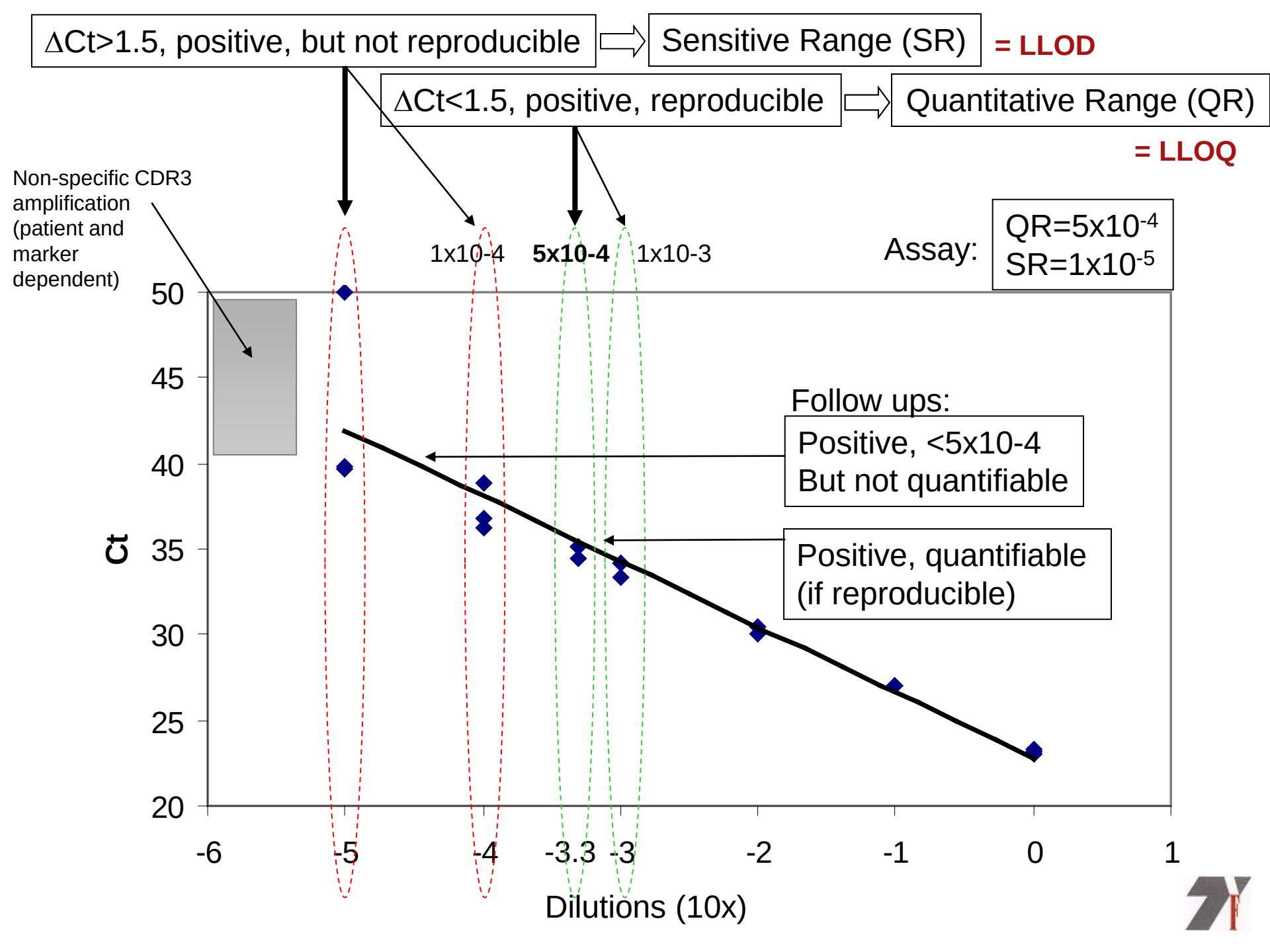
ΔCt>1.5, positive, but not reproducible
Sensitive Range (SR)
= LLOD
ΔCt<1.5, positive, reproducible
Quantitative Range (QR)
= LLOQ
Non-specific CDR3 amplification (patient and marker dependent)
1x10-4
5x10-4
1x10-3
Assay:
QR=5x10-4
SR=1x10-5
Follow ups:
Positive, <5x10-4
But not quantifiable
Positive, quantifiable
(if reproducible)
Ct
50
45
40
35
30
25
20
-6
-5
-4
-3.3
-3
-2
-1
0
1
Dilutions (10x)